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International Study on *Artemia*[1] III. The use of Coulter Counter® equipment for the biometrical analysis of *Artemia* cysts. Methodology and mathematics

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Abstract

Geographical strains of the brine shrimp, *Artemia*, can be characterized by the diameter of their cysts, their cyst volume and chorion thickness. This paper describes the routine procedure that has been worked out for an accurate size-analysis of *Artemia* cyst batches using Coulter Counter® equipment.

Introduction

Within the framework of the international and interdisciplinary study on various geographical strains of the brine shrimp, *Artemia*, the Artemia Reference Center contributes to the overall characterization of over 50 different geographical strains (Sorgeloos *et al.*, 1976): among other studies this task includes a detailed quantitative analysis of the size of the cysts, as characterized by their diameter, volume and chorion thickness. From preliminary studies performed in our laboratory (Claus *et al.*, 1977) it appeared indeed that there are considerable size differences from one geographical strain to another. A statistical analysis of the data gathered is, however, necessary in order to check the size constancy within batches from the same geographical origin and the size differences between genotypically or phenotypically identical or different strains.

For this purpose we have worked out a routine procedure to measure processed *Artemia* cysts using Coulter Counter® equipment and to statistically analyse the data obtained.

[1] International interdisciplinary study on *Artemia* strains coordinated by the *Artemia* Reference Center, State University of Ghent, Belgium.

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Preparation of the cysts

Since most of the cyst-batches, which we have received from all over the world, contain a certain amount of debris (sand, empty shells, etc.), the samples to be analyzed (1-2 g per strain) are subjected to the washing and cleaning procedure as described by Sorgeloos *et al.* (1978). The remaining full cysts are hydrated for 2 hr at 30 °C in cylindrical-conical tubes containing 100 ml electrolyte solution filtered on a 0.2 μm filter. This solution consists of natural seawater diluted with distilled water to a salinity of 10 ‰, and 1% of lugol's solution. The addition of lugol's solution inhibits the metabolic activity within the cyst embryo so that only a physical swelling occurs. During the 2 hr hydration period, the cysts are kept in suspension by a continuous aeration from the bottom of the tube. One ml of lugol's solution is then added, and the cyst suspension is poured into a 250 ml container, stoppered, and stored at room temperature in darkness, in order to maintain the activity of the lugol.

Microscopic measurements revealed that for an at random sample of four strains, the maximum hydration volume is reached within 2 hr of incubation. Nevertheless the cysts are kept for a total of 24 hr in the hydration medium since D'Agostino (1965) and Collins (personal communication) reported that for some strains from the USA more than 2 hr are needed for full hydration.

Previous work in our laboratory had already shown that neither the addition of the lugol's solution – which allows to store the cysts for a period of several weeks – nor the storage temperature have a significant influence on the final volume of the cysts.

For size analysis on decapsulated cysts 1-2 cyst material is processed according to the procedure described by Bruggeman *et al.* (1979). The decapsulation treatment is followed under the microscope to verify that no parts of the chorion are left. The broken cysts and the light debris are removed according to the flotation procedure outlined by Sorgeloos *et al.* (1978). The remaining cyst-product is then resuspended in a brine solution in a cylindrical-conical tube. After 5 min the heavy debris has settled and is siphoned off. The intact embryos are finally hydrated and prepared for size analysis following the hydration procedure described above.

Size-analysis with Coulter Counter® equipment

The measurements are performed with a counter ZB equipped with a channelyzer C-1000 and a P64 X-Y recorder.

One hour before the measurements the cysts are filtered off on a 110 μm screen. The lugol is washed out with electrolyte solution and the cysts resuspended in 50 ml electrolyte solution. Subsamples of 4 ml are taken and transferred to the measuring beaker.

The operational settings on the Coulter Counter® are as follows :

- tube orifice : 560 μm
- I/aperture current : 4
- I/amplification : 16
- base channel threshold setting : 10
- window width setting : 100
- count range : 400

- count control switch : stop at full scale
- edit : off

Since it appeared that the reference channel number for a specific volume-range is affected by varying vacuum pressures in the ZB measuring unit, the vacuum pressure is kept constant at 20×10^3 Pa. Prior to start a series of measurements, the calibration for channel-size analysis has to be performed following the procedure outlined in the Instruction Manual for the Coulter Channelyzer C-1000, (Coulter Electronics Ltd, 1973).

Data analysis

The numerical data obtained with the Coulter Counter® provide a frequency distribution (Table I).

TABLE I

Frequency distribution of the numerical data obtained for *Artemia* cysts from Adelaide (Australia)

Channel no.	Frequency	Channel no.	Frequency
4	2	27	348
5	0	28	273
6	0	29	228
7	0	30	207
8	1	31	118
9	2	32	121
10	2	33	89
11	2	34	56
12	0	35	49
13	9	36	20
14	15	37	35
15	20	38	14
16	40	39	11
17	52	40	10
18	90	41	9
19	146	42	6
20	189	43	4
21	253	44	2
22	337	45	1
23	386	46	13
24	399	47	6
25	397	48	2
26	351	49	3

From these data and the total number of cysts analysed – given by the C-1000 integrator – the mean, variance, and standard deviation of the distribution can be calculated.

The distribution can also be represented graphically by an X-Y plot on the recorder (Fig. 1).

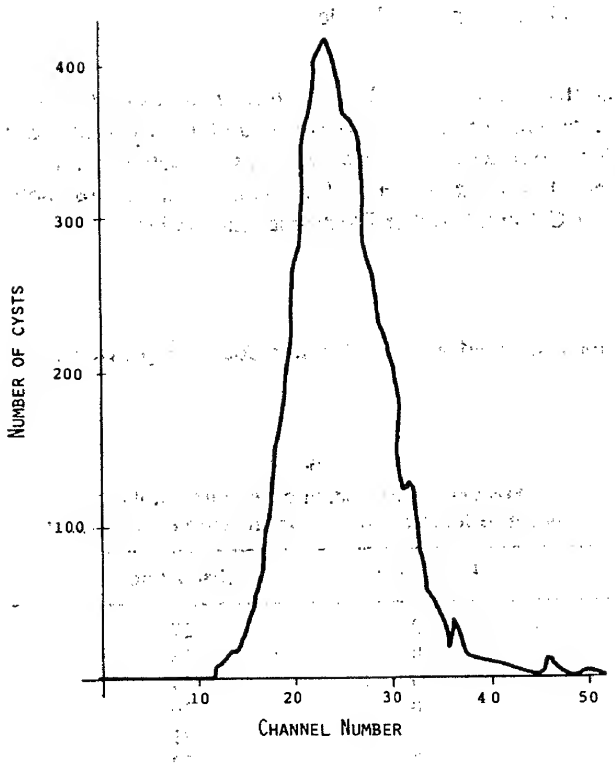

FIG. 1. Size distribution of *Artemia* cysts from Adelaide (Australia).

The specific individual cyst volume per channel (V_N) can be calculated with the following formula from the Instruction Manual for the Coulter Channelyzer C-1000 (Coulter Electronics Ltd, 1973):

$$[(C_N \times \frac{WW}{100}) + BCT] \times TF = V_N \quad (1)$$

where C_N : channel of a particular size class
WW : window width setting = 100
BCT : base channel threshold setting = 10
TF : threshold factor (see further)

C_N and V_N are in our case the specific channel number and the specific volume in cubic microns of the mean of the frequency distribution curve. The threshold factor (TF), which has to be determined for each new series of analyses, is determined with the aid of a sample of calibration material of well known volume. Since there is no specific calibration material available on the market within the size-range of *Artemia* cysts, we decided to use the commercial cyst batch San Francisco Bay, no 288-2596 to calibrate the Coulter Counter®. The mean volume V_C of this cyst sample was assessed from over 600 microscopic measurements.

The threshold factor can now be calculated as follows :

$$TF = \frac{V_C}{C_C + BCT} \tag{2}$$

where V_C : volume in μm^3 of the calibration material
C_C : channel number of the mean of the distribution for the calibration material

The cyst diameter is calculated from the cyst volume using the formula :

$$D = \sqrt[3]{\frac{6V}{\pi}} \tag{3}$$

To calculate the variance σ^2_v and the standard deviation σ_v of the volume, the variance σ^2 and the standard deviation σ of the distribution – given as channels – are multiplied with respectively the square TF value and the TF value.

$$\sigma^2_v = \sigma^2 \times (TF)^2 \tag{4}$$

$$\sigma_v = \sigma \times TF \tag{5}$$

Using $\sigma^2\{f(x)\} = (\frac{\delta f}{\delta x})^2 . \sigma^2 x$ with $f(x) = d = \sqrt[3]{\frac{6V}{\pi}}$
$x = V$

the variance of the diameter is given by the formula :

$$\sigma^2_d = \sigma^2_v \left(\frac{2}{9\pi V^2}\right)^{2/3} \tag{6}$$

Upon comparison of the mean volumes and diameters of replicate cyst samples from the same batch, significant differences were found at the 0.01 level. Statistical analysis of the data obtained following the methods of the maximum likelihood and the weighed sum of squared deviates revealed that the real variance of the mean is about 10 times higher than the estimated value. This appears to be due to the ± 1 % accuracy of the channelyzer. As a consequence 10 measurements were performed for each batch in order to be able to compare the Coulter data on a statistical basis. This way a mean channelnumber $\bar{C}_N$ with variance $\sigma^2_{\bar{C}_N}$ can be determined out of the 10 replicates of each batch. The TF value is also calculated from five replicates. Since the TF value is not constant, but determined from the mean channelnumber $\bar{C}_N$ of five replicates, this should be included in the calculation of the variances.

The mean volume of a batch is then given by :

$$\bar{V} = (\bar{C}_N + BCT) \times TF \tag{7}$$

or

$$\bar{V} = (\bar{C}_N + BCT) \times \frac{\bar{V}_C}{\bar{C}_C + BCT}$$

Using $\sigma^2\{f(x,y)\} = \sigma^2_x \left(\frac{\delta f}{\delta x}\right)^2 + \sigma^2_y \left(\frac{\delta f}{\delta y}\right)^2$ with $f(x,y) = V$
$x = \bar{C}_N$
$y = \bar{C}_C$

$$\text{we obtain : } \sigma^2_v \simeq \sigma^2_{\bar{C}_N} \times (TF)^2 + \sigma^2_{\bar{C}_C} \left[\frac{(\bar{C}_N + BCT)\bar{V}_C}{(\bar{C}_C + BCT)^2}\right]^2 \quad (8)$$

A t-test can be performed to compare the mean volumes of different batches and strains using the data from each analysis for the mean volume $\bar{V}$, its variance and the number of replicates.

In order to compare the mean diameters, the mean and the variance of the diameter has to be calculated using the equations (3) and (6).

Provided data are available for both non decapsulated and decapsulated cysts, one can calculate the volume and the diameter of the chorion (the alveolar and cortical layer as described by Morris and Afzelius (1967) from the differences between the respective values for untreated and decapsulated cysts.

An u-test is used to compare the chorion-volume of two strains :

$$u = \frac{(\bar{V}_1 - \bar{V}_1') - (\bar{V}_2 - \bar{V}_2')}{\sqrt{\frac{s^2_{v1}}{n} + \frac{s^2_{v1'}}{n} + \frac{s^2_{v2}}{n} + \frac{s^2_{v2'}}{n}}} \quad (9)$$

with $\bar{V}_1$ and s^2_{v1} = mean volume and variance for the non decapsulated cyts of the 1st strain
$\bar{V}_1'$ and $s^2_{v1'}$ = mean volume and variance for the decapsulated cysts of the 1st strain
$\bar{V}_2$ and s^2_{v2} = mean volume and variance for the non decapsulated cysts of the 2nd strain
$\bar{V}_2'$ and $s^2_{v2'}$ = mean volume and variance for the decaptulated cysts of the 2nd strain
n = number of replicates.

The calculated value of u has to be compared with the percentage of the Gaussian distribution or the value of t for ∞ degrees of freedom in the Fisher-table. The same formula can be used for the comparison of the chorion diameter of two strains if the mean diameters and the variances of the diameters are calculated.

Practical example

The cyst volume, cyst diameter, chorion volume and chorion thickness have been determined for batches from three geographical strains of *Artemia*.

The channel numbers for the mean of the frequency distribution and the corresponding estimation of the variance for the 10 replicates of untreated and decapsulated cyst samples are summarized in Table II.

Since the strains were not all analyzed on the same day, a slightly different TF value was obtained. Table III contains the channel numbers for the mean of the frequency distribution of the calibration material. Calibration 1 was used for SFBB 2606 untreated, Shark Bay decapsulated and Adelaide decapsulated. Calibration 2 was used for SFBB 2606 decapsulated and calibration 3 was used for Shark Bay untreated and Adelaide untreated. Since the volume of the calibration material is 5 937 913 μm^3 the TF value is given by :

$$TF = \frac{5\,937\,913}{\bar{C}_c + 10}$$

TABLE II

Channel numbers (C_N) for the mean of the frequency distribution and corresponding estimation of the variance (s^2) for the 10 replicates of untreated (A) and decapsulated (B) cyst-samples

San Francisco Bay, California, USA (SFBB 2606)				Shark Bay, Australia				Adelaide, Australia			
A		B		A		B		A		B	
C_N	s^2	C_N	s^2	C_N	s^2	C_N	s^2	C_N	s^2	C_N	s^2
24.3	27.5	18.2	26.9	43.3	36.3	33.5	27.5	25.5	24.8	18.5	16.0
24.5	28.1	18.0	24.6	43.4	33.7	33.7	27.8	25.7	28.6	18.1	15.8
24.6	29.2	18.3	24.6	43.3	37.3	33.3	27.9	25.2	25.4	18.1	14.3
24.6	33.8	17.9	23.2	43.4	37.2	33.3	27.2	25.2	27.1	18.0	13.5
24.5	29.5	18.1	22.9	43.4	35.0	33.0	26.4	25.4	25.1	17.8	14.3
24.7	29.4	18.1	24.0	43.8	35.4	33.5	30.2	24.9	25.1	18.0	14.6
24.7	30.7	18.2	24.1	43.7	36.5	34.4	30.7	25.3	26.0	18.0	14.4
24.7	30.1	18.1	23.9	44.3	36.1	34.3	32.3	25.3	26.1	18.3	14.1
24.5	29.3	18.0	23.8	43.7	35.8	34.0	34.8	24.4	27.0	18.3	14.4
24.3	31.9	18.1	24.0	43.6	35.6	34.1	30.6	25.2	25.5	18.3	14.3
$\bar{C}_N$	$\bar{s}^2$	$\bar{C}_N$	$\bar{s}^2$	$\bar{C}_N$	$\bar{s}^2$	$\bar{C}_N$	$\bar{s}^2$	$\bar{C}_N$	$\bar{s}^2$	$\bar{C}_N$	$\bar{s}^2$
24.54	29.95	18.10	24.20	43.59	35.89	33.71	29.54	25.21	26.07	18.14	14.57

TABLE III

Channel numbers for the mean of the frequency distribution of the calibration material

Calibration 1	Calibration 2	Calibration 3
C_c	C_c	C_c
24.4	24.3	24.9
24.7	24.2	24.9
24.6	24.0	24.2
24.7	23.9	24.8
24.5	24.4	24.7
$\bar{C}_c$ 24.58	24.16	24.70

The data for the cyst volume, cyst diameter, chorion volume, and chorion thickness of the three *Artemia* strains are given in Table IV.

The three figures for the variance $\sigma^2_{\bar{c}_n}$ of the different cyst batches are given in Table V. The variance of the calibration material for calibration 1 is 0.017 ; for calibration 2: 0.043, and for calibration 3: 0.085.

Since the TF values, $\sigma^2_{\bar{C}_N}$, $\sigma^2_{\bar{C}_c}$, $\bar{C}_N$, $\bar{C}_C$ and $\bar{V}_C$ are known, it is possible to calculate σ^2_v and σ^2_d using the equations (8) and (6) ; the data obtained for the different cyst batches are given in Table VI.

A Student t-test reveals that the untreated cysts of Adelaide are significantly larger than the San Francisco Bay cysts at the 0.01 confidence level, whereas the mean diameters of the decapsulated cysts for the San Francisco Bay and Adelaide batches are not significantly different.

TABLE IV

Cyst volume, cyst diameter, chorion volume and chorion thickness of the three *Artemia* strains

	San Francisco Bay		Shark Bay		Adelaide	
	A	B	A	B	A	B
Volume (μm^3)	5 931 036	4 884 511	9 170 374	7 505 633	6 025 170	4 832 060
s	939 738	855 111	1 025 156	933 283	873 723	655 447
Diameter (μm)	224.6	210.5	259.7	242.9	225.8	209.8
s	11.9	12.3	9.7	10.1	10.9	9.5
Chorion volume (μm^3)	1 046 525		1 664 712		1 193 110	
Chorion thickness (μm)	7.05		8.40		8.00	

TABLE V

Variance data of untreated (A) and decapsulated (B) cyst batches

Strain	$\sigma^2_{C_N}$	
	A	B
San Francisco Bay	0.02267	0.01333
Shark Bay	0.09433	0.22100
Adelaide	0.12544	0.04267

TABLE VI

σ^2_v and σ^2_d for the three *Artemia* strains

	σ^2_v		σ^2_d	
	A	B	A	B
San Francisco Bay	1.16865×10^9	1.28195×10^9	0.1862	0.2646
Shark Bay	8.69890×10^9	7.31732×10^9	0.7751	0.8516
Adelaide	6.23590×10^9	1.59011×10^9	0.9728	0.3329

The cysts from Shark Bay, untreated and decapsulated, are significantly larger than the respective cyst preparations of the two other strains.

An u-test, using equation (9) performed on the date for these strains reveals that at the 0.01 confidence level the chorion volume of the Shark Bay cysts is significantly larger than that of the Adelaide cysts, which in turn have a significantly larger chorion volume than the San Francisco Bay cysts.

With regard to the chorion thickness there appears to be no difference between the Adelaide and the Shark Bay strain. The chorion of the San Francisco Bay cysts seems, however, to be significantly thinner than the corresponding value for the two other strains.

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